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Award Number: DAMD17-97-1-7208

TITLE: Role of the ATM Gene in Bilateral Breast Cancer Following Radiotherapy

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REPORT DATE: August 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Annual (11 Jul 99 - 10 Jul 00)

4. TITLE AND SUBTITLE
Role of the ATM Gene in Bilateral Breast Cancer Following Radiotherapy

5. FUNDING NUMBERS
DAMD17-97-1-7208

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The purpose of this project is to determine whether women who developed bilateral breast cancer following radiotherapy for an initial breast cancer had a higher incidence of heterozygosity for a mutation in the *ATM* gene than either breast cancer patients who did not develop a second breast cancer or bilateral breast cancer patients who did not receive radiotherapy. To accomplish this, during the first three years of this project, DNA samples were isolated from unilateral and bilateral breast cancer patients and screened for *ATM* mutations using a Non-radioisotopic RNase Cleavage-based Assay (NIRCA). However, not a single mutation was discovered from any of these patients which raised a concern as to the sensitivity of this assay. To address this concern, the PI of this project recently purchased an instrument for the performance of denaturing high performance liquid chromatography (DHPLC). This is a high throughput technique in which large numbers of DNA samples can be rapidly screened with a high degree of sensitivity and accuracy. During the fourth year of this study, representing a no-cost extension of this project, all of the DNA samples obtained from the unilateral and bilateral breast cancer patients will be re-screened for *ATM* mutations using DHPLC. We have begun this process with screening eight *ATM* exons and have already found eight patients possessing base sequence alterations that had previously gone undetected using NIRCA.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	26
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Barry S. Roberts 8/4/00

PI - Signature Date

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INTRODUCTION

Ataxia telangiectasia (AT), which results from mutations in both copies of the *ATM* gene, is relatively rare with an incidence of approximately only 1 in 100,000. However, the heterozygous condition which is characterized by a mutation in one copy of the gene, is thought to affect roughly 0.5-1% of the population. These individuals do not suffer from AT, however, they appear to be particularly susceptible to the induction of breast cancer by radiation. The hypothesis being examined in this project is that women who are *ATM* gene carriers and receive radiation therapy, generally as part of a breast conservation treatment, are at increased risk of developing a second primary breast cancer. Hence, the goal of this study is to determine whether radiation exposure resulting from the scattered dose to the contralateral breast is associated with an increased occurrence of bilateral breast cancer among women who are heterozygous for *ATM*. This will be accomplished through screening bilateral breast cancer patients for *ATM* mutations. Confirmation of this hypothesis would have important and direct implications upon patient care in that it would suggest that breast cancer patients should be tested for *ATM* heterozygosity. If found positive, a possible recommendation may be that these women should not be candidates for radiotherapy and other treatment modalities utilized. Alternatively, a more effective block technique with dose reduction to the contralateral breast may be implemented for these patients. This would have the positive impact upon the vast majority of patients that are not *ATM* carriers as they could be assured that their risk for radiation induced breast cancer is extremely low as *ATM* carriers may account for most breast cancers resulting exposure of the contralateral breast to radiation associated with radiotherapy.

BODY

Task 1: Month 1: Identify with Dr. Paul Tartter the bilateral breast cancer patients who fall into the two categories to be used in this study.

This was accomplished using records Dr. Tartter has assembled for bilateral breast cancer patients treated at Mount Sinai over the past 18 years.

Task 2: Month 1: Establish a procedure by which the breast cancer patients will be recruited into the study.

There was relatively easy accessibility to unilateral breast cancer patients as many of these patients were treated in the Radiation Oncology Department and continue to come to Mount Sinai periodically for follow-up visits. Arrangements were made so that when patients selected for this study arrived for their exam, an investigator was available to explain the project to the patients and obtain informed consent for participation in the study following which a nurse would draw a blood sample. A difficulty arose, however, recruiting bilateral breast cancer patients into the study as many of these patients, who were treated as long as 18 years ago, had either died, been lost to follow up or rarely visited Mount Sinai. Therefore, a alternate strategy was developed to access the bilateral patients in which paraffin embedded tissue, preferably lymph node biopsy samples, were obtained for these individuals. A collaboration was established with Dr. Ira Bleiweiss, the pathologist at Mount Sinai responsible for review of all breast cancers. Using this approach, we successfully retrieved paraffin-embedded tissue blocks from 41bilateral patients identified by Dr. Tartter.

Task 3: Month 1: Decide which regions of ATM will be subjected to analysis based upon results available in the literature at the time the project begins

As of this point, there are no specific allelic hotspots or regions of *ATM* which have been identified for mutations. Therefore, the entire coding region of the gene is being examined.

Task 4: Months 2-32: Obtain blood samples from breast cancer patients

Blood samples were obtained from a total of 37 unilateral breast cancer patients. In addition, tissue blocks were retrieved for 41 bilateral breast cancer patients.

Task 5: Month 2: Create appropriate primers for RT-PCR and sequencing

During the period when a procedure to recruit bilateral breast cancer patients into the study was being established, efforts were also initiated to create primers for RT-PCR. As this effort was well underway by the time a decision was made to use genomic DNA obtained from paraffin embedded tissue, it was decided to complete construction of the cDNA primers and use them to screen for *ATM* mutations in unilateral breast cancer patients. The results of this portion of the project have been published and a reprint of this manuscript entitled *"ATM* heterozygosity and breast cancer: screening of 37 breast cancer patients for *ATM* mutations using a non-isotopic RNase cleavage-based assay" has been included in the appendix to this report. In addition, primers were designed and validated for use with genomic DNA to amplify all of the exons that comprise the *ATM* gene.

Task 6: Months 3-36: Isolate mRNA from blood lymphocytes of patients

As described, rather than isolating mRNA from blood samples, genomic DNA was isolated from paraffin-embedded biopsy tissues. To accomplish this, the ONCOR EX-WAX™ DNA Extraction Kit was used. The procedure employed was to first cut 10 sections of 5 μ thickness from a paraffin-embedded lymph node biopsy sample and place them in a 1.5 ml tube. 1 ml of 100% ethanol was added, vortexed 15 sec, centrifuged at 12,000 rpm 3 min, the ethanol removed and the pellet air dried. 150 μl of digestion solution and 50 μl of protein digesting enzyme solution were added and the tube incubated 4-18 hr at 50°. 100 μl of extraction solution was mixed and after 15 sec the tube was centrifuged 10 min at 12,000 rpm. The supernatant was removed by poking the pipette tip through the paraffin layer on top and withdrawing the supernatant, leaving the paraffin and pellet behind. 150 μl of precipitation solution was added to the supernatant, the tube inverted 3 times, 900 μl of 100% ethanol (-20°) added and mixed. After 1 hr the tube was centrifuged for 10 min at 12,000 and the supernatant discarded. The pellet was air dried and 50 μl of resuspension solution added for 1 hr at 50°.

Task 7: Months 3-36: Perform RT-PCR with these samples to amplify in sections the coding region of the *ATM* gene.

RT-PCR was not necessary as genomic DNA samples were used. The first stage PCR reactions were performed using approximately 100-500 ng genomic DNA, 125 μM dNTPs, 250 nM primers, 1 U Taq polymerase in a buffer containing 10 mM Tris.Cl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin and RNase free water to a total volume of 50 μl. The PCR reactions were performed in a Perkin Elmer 9700 Thermal Cycle at 94° for 4 min, followed by 30 cycles of 1 min at 94°, 2 min at 54° and 1.5 min at 72° and one cycle for 8 min at 72°. 10 μl of the first stage PCR product was used in a second series of PCR reactions using the same conditions to achieve further amplification of the region of interest and to add the T7 and SP6 promoters to the PCR products.

Task 8: Months 3-36: Use NIRCA to identify PCR products containing mutations

All *ATM* exons were amplified using PCR for the DNA samples isolated from the bilateral breast cancer patients and screened for mutations using a Non-radioisotopic RNase Cleavage-based Assay (NIRCA). Each second stage PCR product was transcribed in two reaction mixes using 2 μl of the PCR product, 250 nM rNTPs, 2 U of either T7 or SP6 RNA polymerase in a buffer containing 40 mM Tris.Cl, pH 7.5, 7 mM $MgCl_2$, 2 mM spermidine, 25 mM NaCl and 10 mM dithiothreitol and brought to a final volume of 10 μl with RNase free H_2O and incubated for 1 hr at 37°. 10 μl of buffer containing 80% (v/v) formamide, 25 mM NaCl, 2 mM EDTA, pH 8.0 was added to each transcription reaction. The T7 and SP6 reactions were mixed, incubated at 95° for 3 min and allowed to cool at room temperature for 1 min. 4 μl of the duplex reactions were mixed with 16 μl of RNase digestion buffer (10 mM NaCl, 10 mM Tris.Cl, pH 7.5, 1 mM disodium EDTA, pH 8.0) with 0.5 μg/ml RNase A, RNase I and RNase T1 and brought to a final volume of 20 μl. The reaction mixtures were incubated at 37° for 20 min and resolved on a 3% agarose gel run at 100 V for approximately 45 min.

Task 9:Months 3-36: Sequence all PCR products which appear to exhibit mutations

No mutations were identified in any of the DNA samples isolated from either the unilateral or bilateral breast cancer patients and therefore it was not necessary to perform DNA sequencing. However, the inability to detect even a single mutation in this study raised a concern that the assay used for this work, NIRCA, did not possess sufficient sensitivity to detect the small number of mutations that are likely to be present in this breast cancer population. To address this concern, the PI of this project recently obtained a denaturing high performance liquid chromatography

(DHPLC) system from Transgenomic. This purchase was accomplished from internal Mount Sinai funding sources.

Through use of DHPLC, single-base substitutions, small insertions and deletions can be detected in 100-1500 bp DNA fragments by fractionation of heteroduplexes on ion-pair reverse-phase columns. The Wave™ DNA Fragment Analysis System manufactured by Transgenomic, that has been installed in the PI's laboratory and is available for this project, is a complete unit for the automated DHPLC analysis of PCR products in a 96 well plate using a DNASep cartridge specifically designed for separation of DNA fragments. This represents a high throughput technique in which large numbers of DNA samples can be rapidly screened for base sequence alterations.

DHPLC relies upon the physical changes in DNA molecules induced by mismatched heteroduplex formation during reannealing of wild type and mutant DNA. In this method, a portion of a gene is amplified using standard PCR conditions and the products analyzed using DHPLC. Material from a homozygous sample will only form one species, the wild-type homoduplex, which will appear as a single peak on the DHPLC chromatogram. However, when the PCR products produced from a sample heterozygous for a base sequence alteration are heated to 95°C, and then slowly cooled, the DNA strands separate and randomly reanneal to form a mixture of three species; a mutant homoduplex, a heteroduplex and a wild type homoduplex. These will appear ideally as four peaks on a chromatogram, although often only two or three peaks are present as either the two homoduplexes or two heteroduplexes may not be resolvable from each other.

On 6/29/00, Ms. Cheryl Miles was informed that we wished to exercise our option for a one-year no-cost extension of this grant so that the new termination date for this project is 8/10/01. The purpose of this extension is to reanalyze all of the samples obtained from the breast cancer patients using the rapid and sensitive DHPLC instrument to determine whether any mutations went undetected using NIRCA.

This process began during the past month and *ATM* exons 7, 10, 17, 19, 40, 44, 55 and 58 have been screened. We have found eight patients possessing base sequence alterations that had gone undetected using NIRCA. The aberrant chromatograms suggestive of heteroduplex DNA, and therefore samples possessing base sequence alterations, are shown in Figures 1, 3, 5 and 7. It should be noted that the peaks at retention times of 0.5-1 min and 7-8 min are to be ignored as they represent unincorporated nucleotides and the acetonitrile wash, respectively. Although the changes from the wild type profile appear in some cases to be subtle, it should be noted that nearly identical chromatograms were produced for the vast majority of patients possessing wild type sequences so that even a small change in

the chromatogram signaled the presence of a DNA sample with a likely base sequence alteration.

In each case, the particular exon for each putative heterozygote was subjected to automated sequencing and the traces shown in Figures 2, 4, 6 and 8. These results confirmed that each of the DHPLC variants was heterozygous for an *ATM* base sequence alteration, thus demonstrating that at least in this limited sample, DHPLC had a 100% true positive rate. The nature of each base sequence alteration is listed in Table 1. We are in the process of reaching a determination as to which of the base sequence alterations are likely to affect ATM protein function and thus determine which patients possess *ATM* mutations.

Because DHPLC is a relatively rapid assay suitable for screening large numbers of samples, we will be able to complete the screening of the entire *ATM* gene for all of the unilateral and bilateral breast cancer patients during the course of the no-cost extension of this project over the following year.

KEY RESEARCH ACCOMPLISHMENTS

- Through use of DHPLC, base sequence alterations in the *ATM* gene have been discovered in breast cancer patients.

REPORTABLE OUTCOMES

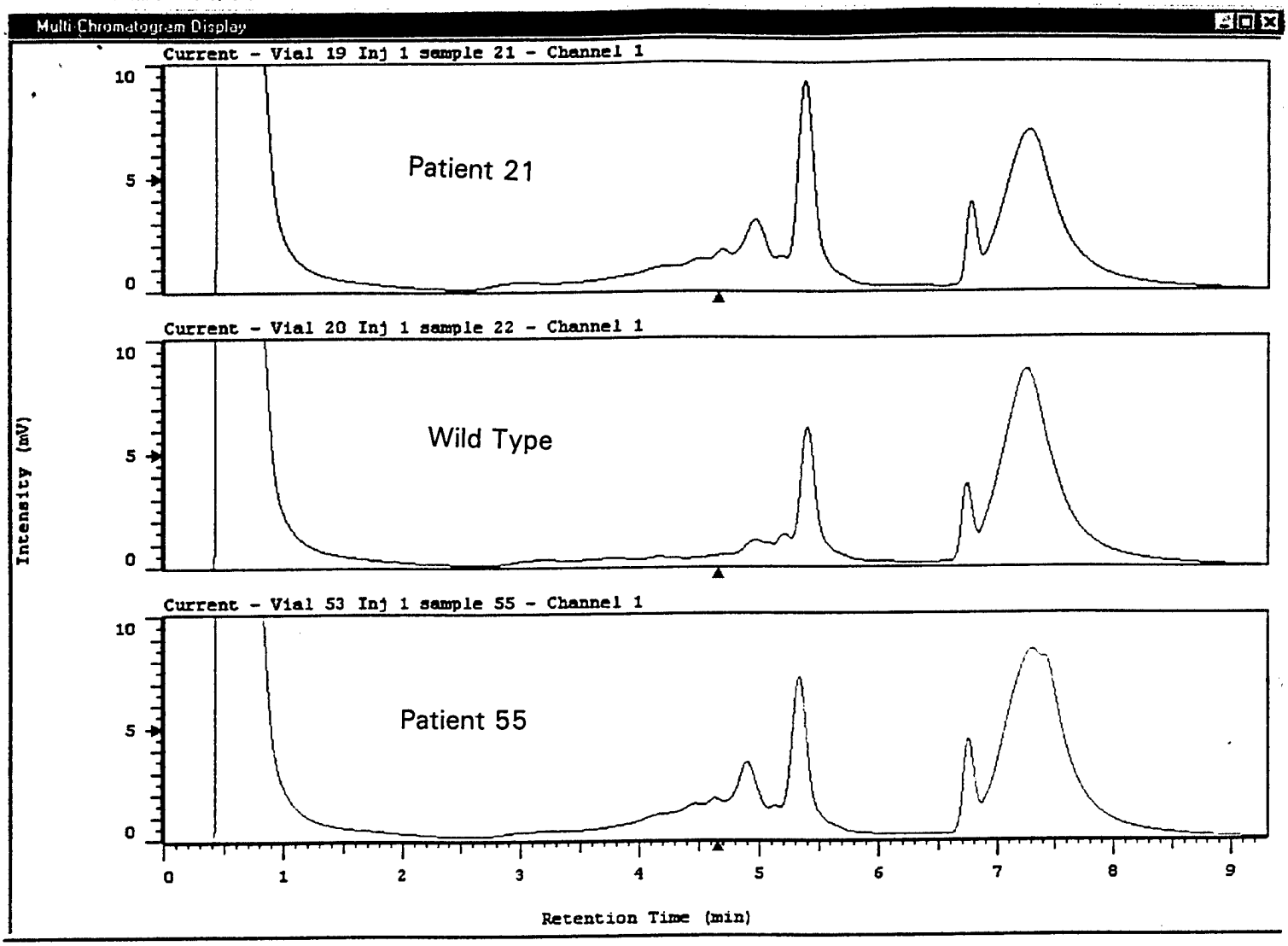
Drumea, Karen C., Eva Levine, Jonine Bernstein, Brenda Shank, Sheryl Green, Lynda Mandell, Joan Cropley, Juliette Obropta, Irene Braccia, Amy Krupnik and Barry S. Rosenstein. *ATM* Heterozygosity and Breast Cancer: Screening of 37 Breast Cancer Patients for *ATM* Mutations Using a Non-Isotopic RNase Cleavage-Based Assay. *Breast Cancer Research and Treatment* 61:79-85, 2000.

CONCLUSIONS

Using DHPLC, we have discovered that roughly 15% of the breast cancer patients tested possess *ATM* base sequence alterations in the roughly 13% of the coding region of the *ATM* gene that has been screened up until this time. Although preliminary, these results are suggestive that a substantial percentage of breast cancer patients in this study will prove to be carriers of *ATM* base sequence alterations, although many may represent polymorphisms that would not be expected to significantly alter protein function.

TABLE 1. *ATM* Base Sequence Alterations

Patient	Exon	Nucleotide	Base Change	Amino Acid Change
15	17	IVS16-17	insT	
21	7	378	T→A	asp→glu
22	19	2752	T→C	phe→leu
25	40	IVS40 + 26	C→T	
48	19	2752	T→C	phe→leu
50	40	IVS40 + 26	C→T	
54	17	2362	A→C	ser→arg
55	7	378	T→A	asp→glu



EXON 7

FIGURE 1

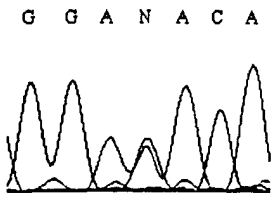
Exon 7

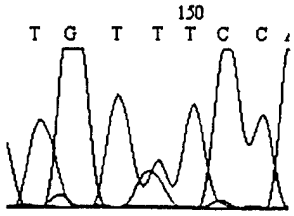
Forward Primer

Reverse Primer

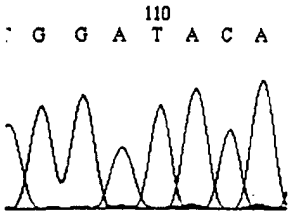
Patient 21

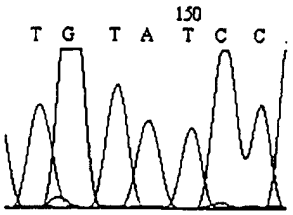
(mutated sequence)





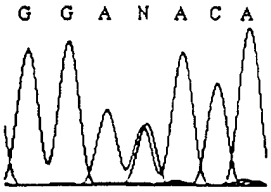
Wild-type sequence





Patient 55

(mutated sequence)



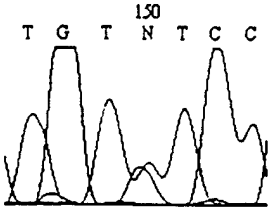
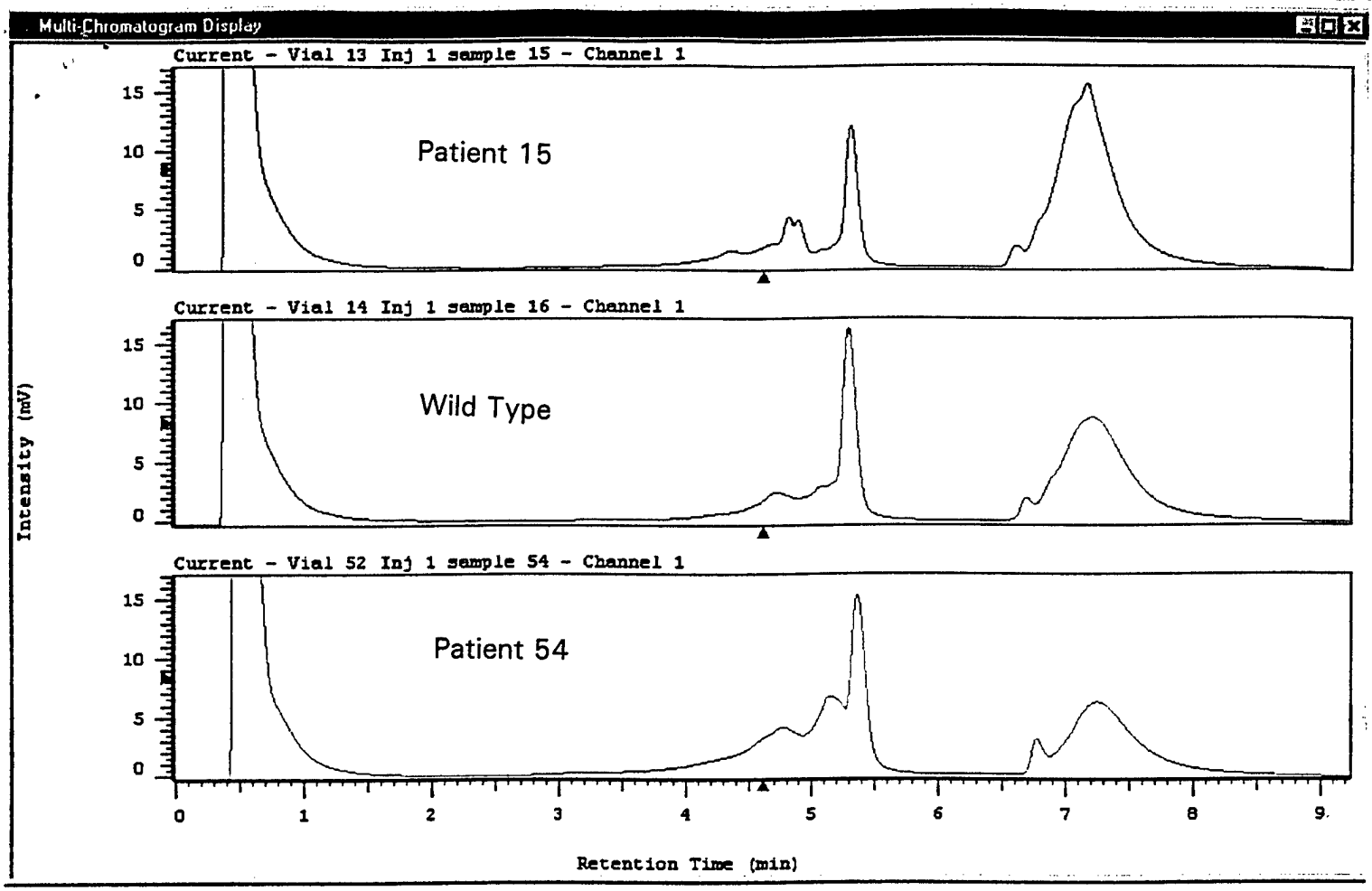


FIGURE 2



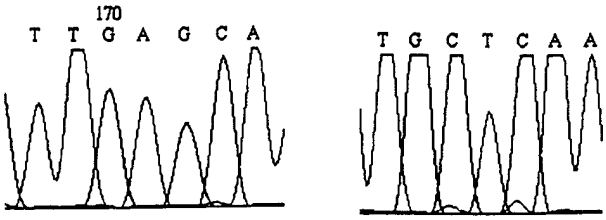
EXON 17

FIGURE 3

Exon 17

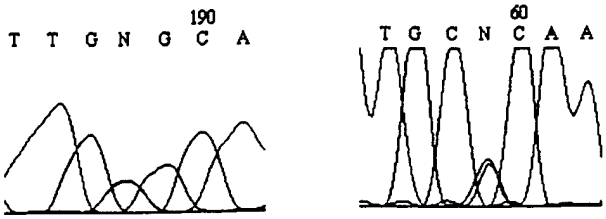
Forward Primer | Reverse Primer

Wild-type Sequence



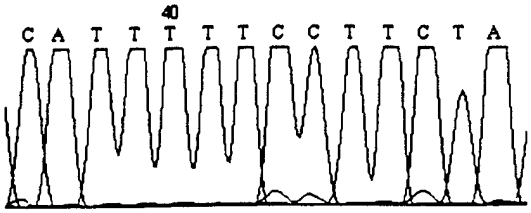
Patient 54

(Mutated Sequence)



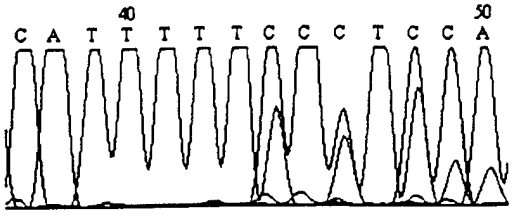
Forward Primer

Wild-type sequence



Patient 15

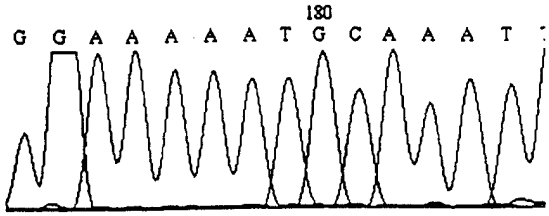
(mutated sequence)



Wild-type trace C A T T T T T C C T T C T A
Insertion trace C A T T T T T *T* C C T T C T

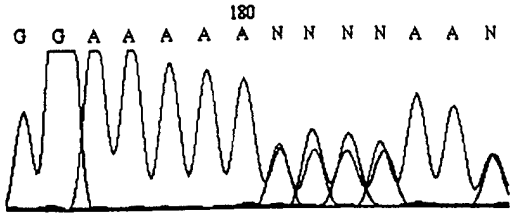
Reverse Primer

Wild-type sequence



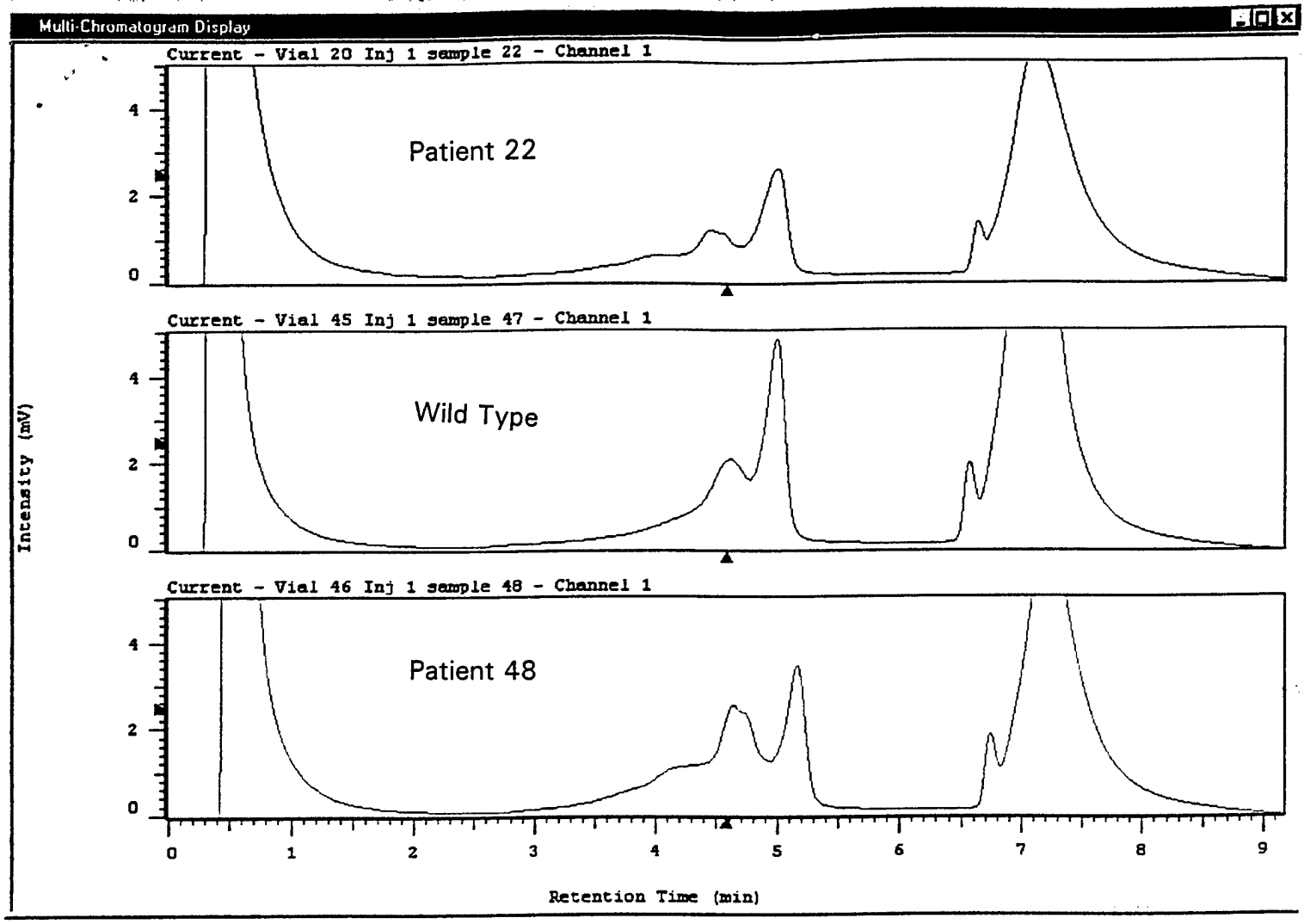
Patient 15

(mutated sequence)



Wild-type trace G G A A A A A T G C A A A T
Insertion trace G G A A A A A *A* T G C A A A

FIGURE 4



EXON 19

FIGURE 5

Exon 19

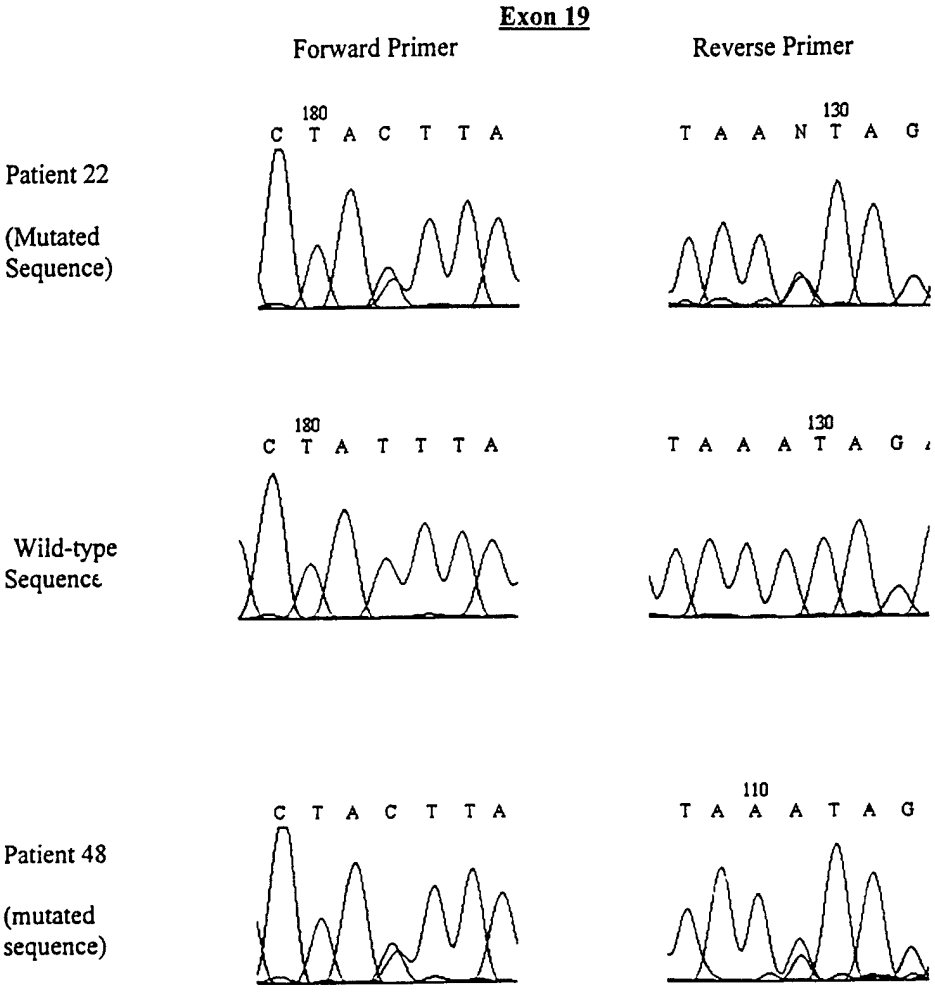
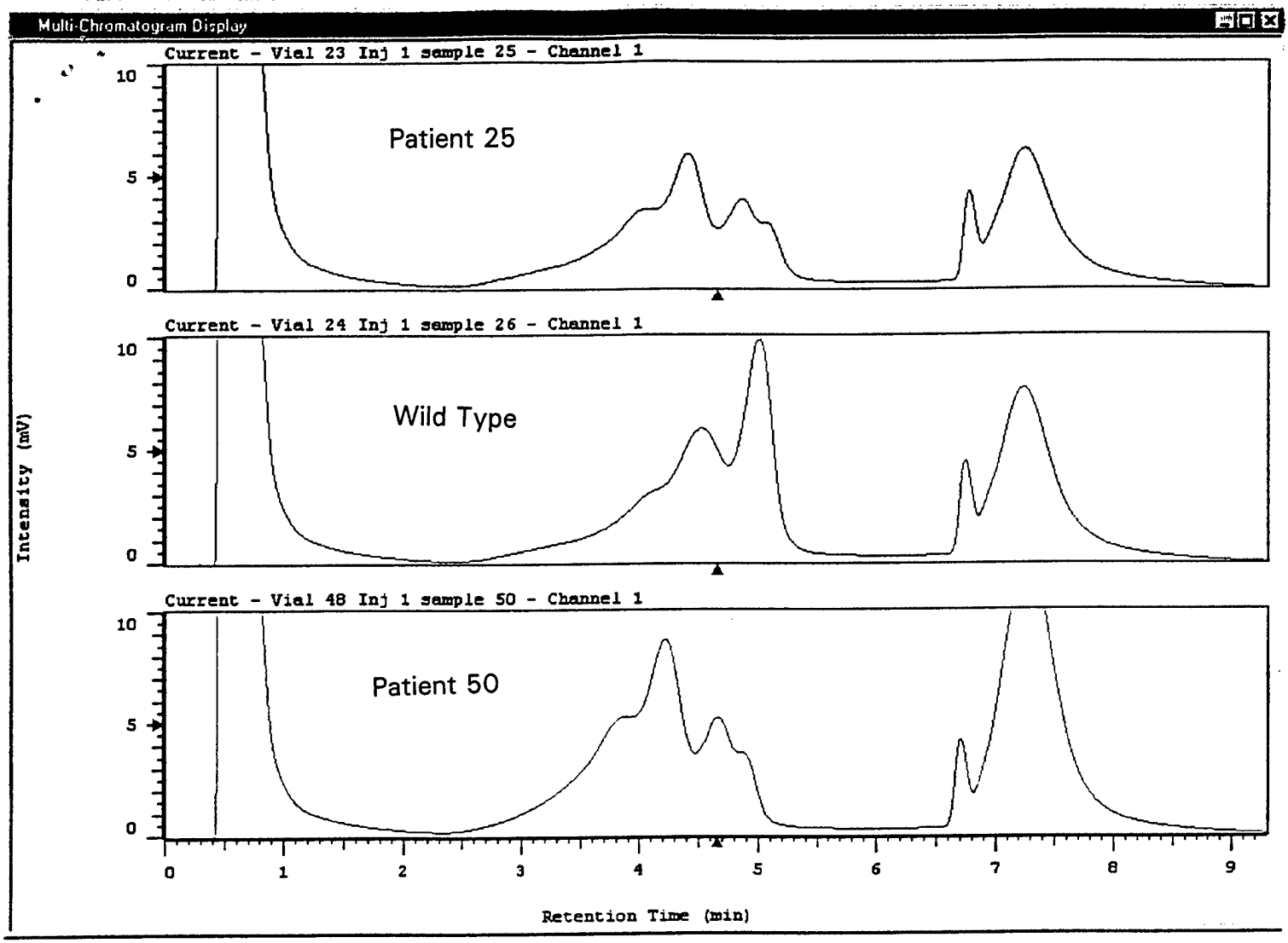


FIGURE 6



EXON 40

FIGURE 7

Exon 40

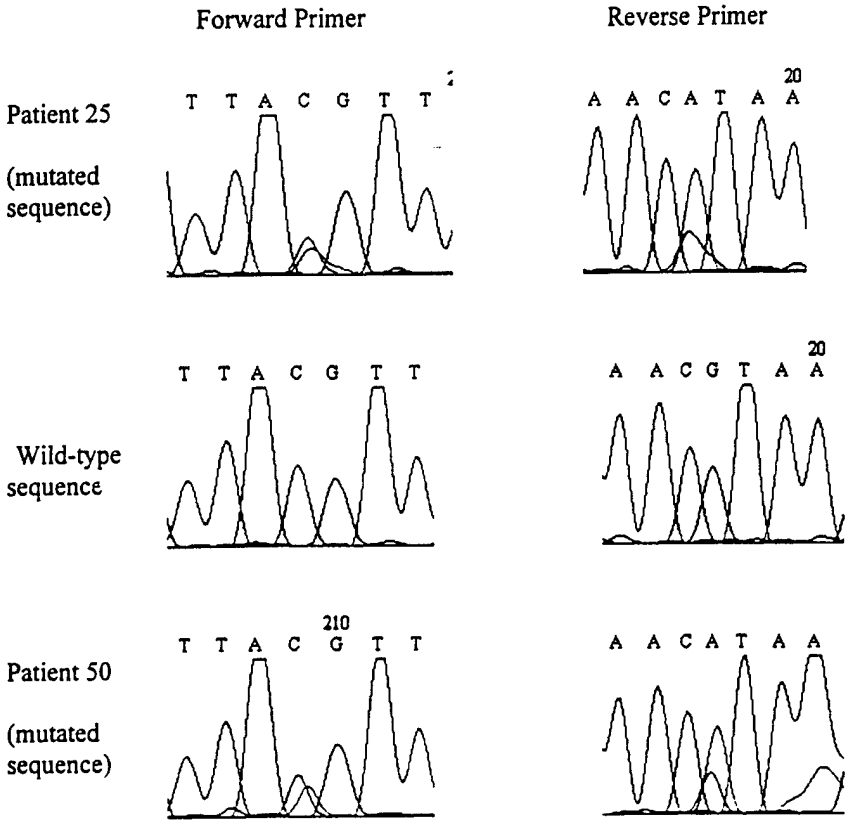


FIGURE 8

Breast Cancer Research and Treatment **61:** 79–85, 2000.

ATM heterozygosity and breast cancer: screening of 37 breast cancer patients for *ATM* mutations using a non-isotopic RNase cleavage-based assay *

Karen C. Drumea[1,4], Eva Levine[1], Jonine Bernstein[2], Brenda Shank[1], Sheryl Green[1], Edward Kaplan[5], Lynda Mandell[1], Joan Cropley[1], Juliette Obropta[1], Irene Braccia[1], Amy Krupnik[1], and Barry S. Rosenstein[1,2,3]

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Key words: ataxia telangiectasia, *ATM*, breast cancer, mutation screening

Summary

Based upon the results of several epidemiologic studies, it has been suggested that women who are carriers for a mutation in the ataxia telangiectasia-mutated (*ATM*) gene are susceptible for the development of breast cancer. Therefore, 37 consecutive breast cancer patients were screened for the presence of a germline *ATM* mutation using a non-isotopic RNase cleavage-based assay (NIRCA). This paper reports the first use of NIRCA for detection of *ATM* mutations in breast cancer patients. Using this assay, no *ATM* mutations were found in our patient population. This result is similar to the findings of other studies that have employed approaches complementary to NIRCA.

Introduction

Ataxia-telangiectasia (AT) is an autosomal recessive disorder characterized by progressive cerebellar degeneration with ataxia, conjunctival telangiectasias, immunodeficiency, chromosomal instability, radiation sensitivity and cancer predisposition [1]. The cancer risk exhibited by AT patients is believed to be approximately 60–180 times higher than that of the general population [2], consisting primarily of lymphomas and leukemias. Ataxia telangiectasia-mutated (*ATM*), the gene whose alteration results in AT, has been cloned and mapped to chromosome 11 q22-23 [3, 4]. *ATM*, which is expressed in a wide variety of tissues, is approximately 150 kb in length possessing 66 exons with a 12 kb transcript. The open reading frame of 9168 nucleotides encodes a nuclear phosphoprotein of 3056 amino acids. Most AT patients are compound heterozygotes with the location of mutations spread throughout the genome [5]. Concannon and Gatti [6] found that 72% of 78 unique mutations demonstrated in AT patients would be predicted to produce either a truncated protein or no product.

A hallmark of this disease is great sensitivity to radiation as AT patients who were exposed to conventional radiation doses for cancer treatment developed devastating tissue necrosis [7]. The basis for this radiosensitivity has long been the subject of investigation that advanced significantly with cloning of the *ATM* gene. It has been demonstrated that the *ATM* protein belongs to an expanding family of large eukaryotic proteins involved in intracellular signaling, cell cycle control, DNA repair and recombination in response to DNA damage. This family of proteins has been grouped together with *ATM* primarily based upon the strong homology of their carboxyl termini to the 100 kDa catalytic subunit of the mammalian signal transduction mediator phosphatidylinositol-3 (PI-3) kinase. PI-3 kinases appear to participate in many

* This paper is dedicated to the memory of Eva Levine, whose extraordinary sensitivity and devotion to her patients was an inspiration to her colleagues and will forever he missed.

cellular processes, including insulin-dependent glucose transport, growth factor responses and cellular differentiation [8–10].

The worldwide incidence of AT is estimated at 1:40,000–1:100,000 live births [11, 12]. In contrast to the relative rarity of this disease resulting from *ATM* homozygosity, heterozygosity is far more common and it has been estimated that approximately 1% of the population possesses a mutation in one copy of this gene [13]. These heterozygotes do not manifest any outward radiation sensitivity, but, cells derived from individuals who are carriers for a mutation in this gene exhibit a slightly increased radiation sensitivity [14, 15].

Based upon the results of epidemiologic studies, it has been suggested that *ATM* carriers have a 2–6 fold greater risk for cancer development and are particularly predisposed to breast cancer compared with non-carriers [16–18]. It has been estimated that 9–18% of all women with breast cancer may be *ATM* heterozygotes [19]. To address this issue, Easton [20] performed a pooled analysis of four studies [16, 17, 19, 21] from which it can be suggested that AT heterozygotes are more prone to breast cancer and could account for up to 13% of breast cancer cases, with 3.8% being the best estimate. However, recent studies have cast a doubt upon this suggestion as the level of mutations detected among breast cancer patients has been consistent with only the 1% frequency estimated for the general population [22–24]. In addition, *ATM* mutations have not been detected in breast cancer patients who developed unusually severe responses to radiotherapy indicating that *ATM* heterozygosity does not result in acute complications associated with this treatment [25–27].

All of the studies performed to date have been accomplished using either the protein truncation test (PTT), restriction-endonuclease fingerprinting (REF) or single strand conformation polymorphism (SSCP). However, it is important to note that none of these techniques is capable of detecting all mutations and it is always essential to use a series of screening methods to locate most mutations in a population. It is, therefore, important to employ a complementary assay with a different basis to identify mutations which had possibly gone undetected using other techniques. NIRCA represents such an alternative approach. Hence, the purpose of this study was to determine the prevalence of germlme *ATM* mutations in breast cancer patients using NIRCA.

Methods

Patients

Three milliliter of peripheral venous blood samples were obtained from a hospital-based series of 37 unselected patients diagnosed with primary breast cancer treated with radiation therapy with or without chemotherapy between 1992 and 1997. The nature of the project was discussed with each patient and signed informed consent was obtained prior to obtaining each blood sample. Patient characteristics were obtained by chart review and are presented in Table 1.

Table 1. Breast cancer patient characteristics

Patient characteristics	Number of patients (%)
Age Group (years)	
Under 40	2 (5.5)
40–59	14(37.8)
60–80	20 (54)
Over 80	1(2.7)
Median Age = 62	
Ethnic group	
Caucasian[a]	20 (54)
Hispanic	13 (35.2)
Black	3(8.1)
Asian	1(2.7)
Menopausal status	
Pre- and peri-menopausal	9 (24.3)
Postmenopausal	28 (75.7)
Family history of cancer	
Breast[b]	13 (35.2)
Other site	9 (24.3)
None	15 (40.5)
Stage of disease	
Stage 0	3 (8.1)
Stage I	27 (80)
Stage II	6 (16.2)
Stage III	1 (2.7)
Histology	
Intraductal	3 (8.1)
Infiltrating duct	26 (70.1)
Infiltrating lobular	2 (5.5)
Tubular	1 (2.7)
Tubulo-lobular	3 (8.1)
Adenocarcinoma	2 (5.5)

[a] 11 patients of Ashkenazi Jewish background.
[b] 7 patients with first-degree relatives.

RNA extraction and cDNA synthesis

Lymphocytes were isolated by Ficol-plaque separation. 1.5 ml Ficol was added to 3 ml of blood and centrifuged at 12000 rpm for 20 min at room temperature. The lymphocytes layer was collected, washed with 10 ml PBS and centrifuged for 10 min, room temperature, at 12,000 rpm. To isolate RNA from the lymphocytes, 2 ml RNAzol^{TM} (Cinna/TEL-TEST) was added to the cells, followed by addition of 100 μl of chloroform and centrifuged at 4°C for 15 min at 12,000 rpm. The supernatant was transferred to a 1.5 ml tube and an equal volume of isopropanol added to the supernatant. The tube was held at −20°C overnight and centrifuged 15 min at 4°C. The supernatant was removed and the pellet washed with 500 μl of 70% ethanol. After centrifugation at 4°C, 12,000 rpm, for 8 min, the supernatant was removed and the pellet washed with 200 μl of 70% ethanol and centrifuged once more at 4°C, 12,000 rpm speed, for 8 min. The supernatant was removed, the pellet air-dried and 30 μl RNase free H_2O added.

cDNA was synthesized using the isolated RNA as a template by combining 20 μl RNA solution with 4 μl random primers. The mixture was heated to 70°C for 10 min and quickly chilled on ice. After a brief centrifugation, 4 μl of 5 × first strand buffer, 2 μl 0.1 M DTT and 1 μl 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP and dTTP at pH 7.0) were added and the mixture incubated 2 min at 42°C. Two micro litre (200 units) SUPERSCRIPT^{TM} was added, the mixture incubated 50 min at 42°C and then 70°C for 15 min. To remove RNA complementary to the cDNA, the reaction was treated with 2 units *E. coli* RNase H and incubated at 37°C for 20 min.

PCR conditions and primers

Segments of the *ATM* gene were amplified from aliquots of the cDNA template using sets of primer pairs. The primers were designed with the aid of Oligo-5 software (NBI, Plymouth, MN) and are listed in Table 2 (first stage primers) and Table 3 (second stage primers). The *ATM* open reading frame was amplified in a series of 12 overlapping fragments that were approximately 1 kb in size. The product of each PCR reaction was then used as a template for a second reaction to further amplify this region of the gene. In addition, the primers were designed so that sequences for the *T7* and *SP6* promoters were incorporated into the 5′ end of each sense and antisense primer, respectively. Two microlitre of cDNA was used in a 50 μl PCR reaction using 250 nM first stage primers, 125 μM dNTPs, 1 U Taq polymerase in a buffer containing 10 mM Tris-Cl at pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin. The PCR reactions were performed in a Perkin Elmer 9700 ThermalCycler with the following settings: 94°C for 4 min, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, and one cycle for 7 min at 72°C. One microlitre of first stage PCR product was used in the nested PCR reactions using the same conditions, with second stage primers.

Table 2. Sequences of first stage *ATM* primers

Fragment number	Bases amplified	Primer sequence
1-Sense	5–906	GAGGAGTCGGGATCTGC
1 -Antisense		GACAGCCAAAGTCTTGAG
2-Sense	695–1676	CTGTGTACTTCAGGCTCT
2-Antisense		GCTTGTATTTGCTCAGAA
3-Sense	1544–2509	CACCATATGTGTTACGAT
3-Antisense		TCATATTTCTCAAGGAAC
4-Sense	2329–3298	ACTCTTGTCCGGTGTTC
4-Antisense		AGGGCCATTCTTACAGA
5-Sense	2978–3977	TAATTGATTCTAGCACGC
5-Antisense		CTTCTAATCACCAGATGT
6-Sense	3877–4850	AACTTATCTTCTTTTCC
6-Antisense		AATCGTGATATAGAGGT
7-Sense	4710–5682	TTACTGTAAGGATGCTC
7-Antisense		AGTCAGTTTTCACTTCA
8-Sense	5559–6534	TGATATAAATCTGTGGA
8-Antisense		TTCTACTTCTTTGCTG
9-Sense	6407–7353	TCTGCCATATTCTTTCC
9-Antisense		AACCGGGCTAATGAG
10-Sense	7193–8172	CAGAATGTCTGAGGGT
10-Antisense		TAGTAGGGACAACAACA
11-Sense	8047–8952	GTTGAGGCACTTTGTGA
11 -Antisense		AGACACCTTCAACACCC
12-Sense	8409–9353	GAGAAACACGGAAACTA
12-Antisense		TACTGAAGATCACACCC

Non-isotopic RNase cleavage assay (NIRCA)

The MisMatch Detect II Non-Isotopic RNase Cleavage Assay Kit (Ambion, Austin, TX) was used to screen the samples for *ATM* mutations [28, 29]. Each second stage PCR product was transcribed in two reaction mixes using 4 μl of PCR product, 250 nM rNTPs, 1 U of either T7 or SP6 RNA polymerase in a buffer containing 40 mM Tris-Cl, pH 7.5, 7 mM $MgCl_2$,

2 mM spermidine, 25 mM NaCl and 10 mM dithiothreitol and brought to a final volume of 12 µl with RNase free H_2O. Following incubation for 90 min at 37°C, 6 µl of buffer containing 80% formamide, 25 mM NaCl, 2 mM EDTA, pH 8.0, was added to each transcription reaction. The T7 and SP6 reactions were mixed, incubated at 95°C for 5 min and allowed to cool at room temperature for 5 min. The duplex reactions of 4.5 µl were mixed with 13.5 µl of RNase digestion buffer (10 mM NaCl, 10 mM Tris-Cl, pH 7.5, 1 mM disodium EDTA, pH 8.0) with RNase 2 and RNase 1 (from *E. coli*) at a concentration of 1:100 (0.5 µg/ml) and 1:200, respectively. The reaction mixtures were incubated at 37°C for 25 min and resolved on a 2% agarose gel run at 100 V for approximately 45 min.

Table 3. Sequences of second stage ATM primers

Fragment number	Bases amplified	Primer sequence[a]
1-T7	37–790	TAATCGACTCACTATAGGG
		CGGTTGATACTACTTTG
1-SP6		ATTTAGGTGACACTATAGAA
		TTTAATCCGTCAGTCT
2-T7	755–1632	TAATCGACTCACTATAGGA
		ATAATTCATGCTGTTAC
2-SP6		ATTTAGGTGACACTATAGAA
		TTTTATTCCAGAGTTT
3-T7	1570–2425	TAATCGACTCACTATAGGA
		AGTTGCATTGTGTCAAG
3-SP6		ATTTAGGTGACACTATAGGA
		GTTGGCTTTCTGGAA
4-T7	2368–3247	TAATCGACTCACTATAGGG
		CTGCTACTGTTACA
4-SP6		ATTTAGGTGACACTATAGAA
		TGCTCCAATTACTGT
5-T7	3055–3944	TAATCGACTCACTATAGGA
		GAAGAGTACCCCTTGC
5-SP6		ATTTAGGTGACACTATAGGA
		GATGTGGAATCAAAACCTTAT
6-T7	3911–4796	TAATCGACTCACTATAGGA
		CACAAATATTGAGGAT
6-S P6		ATTTAGGTGACACTATAGAA
		GTCCAATACCTGTT
7-T7	4782–5644	TAATCGACTCACTATAGGG
		TGGAGGTTCAGA
7-SP6		ATTTAGGTGACACTATAGAA
		GAATTTCACATTTTGT
8-T7	5607–6500	TAATCGACTCACTATAGGA
		AAGACACTGACTTGTG
8-SP6		ATTTAGGTGACACTATAGAA
		CTGCATATTCCTCC
9-T7	6451–7285	TAATCGACTCACTATAGGA
		ATAAAGACTGGTGTCC
9-S P6		ATTTAGGTGACACTATAGAA
		TTTCCAGCAACTTC
10-T7	7213–8113	TAATCGACTCACTATAGGG
		CAACTGGTTAGC
10-SP6		ATTTAGGTGACACTATAGAA
		TATTTATGCCTTTTCT
11-T7	8082–8669	TAATCGACTCACTATAGGA
		AACTTAGATGCCACTC
11-SP6		ATTTAGGTGACACTATAGGA
		AAACTGGTTGAA
12-T7	8598–9328	TAATCGACTCACTATAGGA
		AAATGATGGAGGTGC
12-SP6		ATTTAGGTGACACTATAGAA
		TCCTGGGAAAAGTCG

[a]The underlined regions indicate the phage promoter sequences. Generally, the first two bases at the 3′ end of the promoter overlapped in each case with the first two bases of the *ATM* fragment.

Results

RNA was isolated from peripheral blood lymphocytes of 37 breast cancer patients in order to detect possible *ATM* mutations. The characteristics of the subjects are presented in Table 1. cDNA copies of the extracted mRNA were produced for each sample. This approach was employed, rather than the use of genomic DNA, to avoid analysis of intronic DNA regions. The *ATM* open reading frame was amplified in 12 overlapping segments using nested PCR. The products of the second stage PCR reactions, all roughly 1 kb in size, were subjected to agarose gel electrophoresis and the results obtained for three patients using two sets of primers and a *p53* control displayed in Figure 1.

The PCR products that contained promoters for either the T7 or SP6 RNA polymerase were then transcribed in an *in vitro* system. The convention followed with this assay was linkage of the *T7* promoter to the sense strand of the fragment while the *SP6* promoter was attached to the antisense strand. The RNA product produced from either the sense or antisense strand was hybridized with the complementary RNA strand produced using known normal tissue not possessing an *ATM* mutation. In addition, for each experiment, the

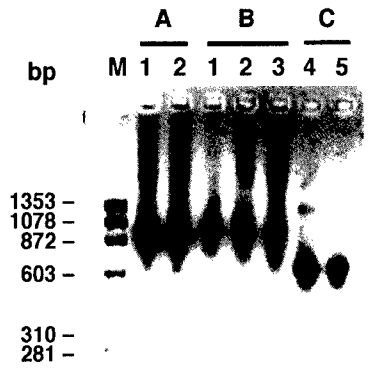

Figure 1. PCR products. Second stage PCR products for DNA isolated from two breast cancer patients (lanes 1 and 2) and one person not diagnosed with breast cancer (lane 3) and amplified with primers for fragments 4 and 6, labeled as A and B, respectively. DNA known not to possess a mutation in the *p*53 gene and a DNA sample with a known mutation are shown in lanes 4 and 5, respectively. These samples were amplified with primers for a DNA fragment encompassing *p*53 exons 5 and 6 and labeled C.

RNA produced from a known mutant and wild type was hybridized with RNA transcribed from a wild type DNA template to serve as positive and negative controls for this assay, respectively.

These duplexes were subjected to agarose gel electrophoresis either prior to (Figure 2) or following digestion (Figure 3) with RNase. The basis of this screening technique is the sensitivity to cleavage with RNase of an RNA duplex containing a mismatched region [28, 29]. This heteroduplex forms when RNA produced from a PCR product possessing a mutation at a particular site is hybridized with RNA produced from a wild type DNA template. As can be seen in Figure 3, there was the appearance of two bands following

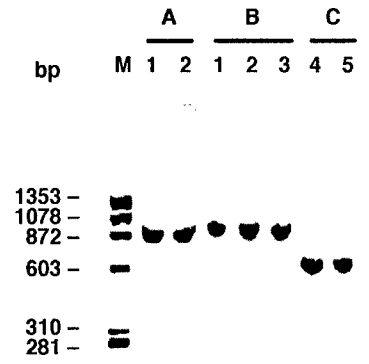

Figure 2. Transcription products – no digestion. RNA products resulting from hybridization of RNA produced from the sense strand of tumor biopsy DNA and RNA synthesized from the antisense strand of DNA isolated from normal human DNA. The lanes are labeled as described in Figure 1.

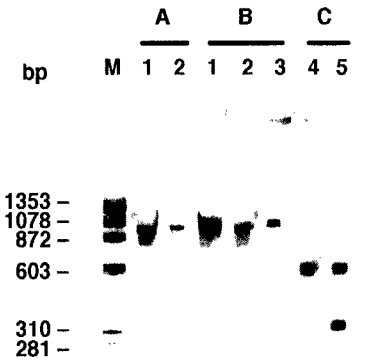

Figure 3. RNase-treated transcription products. Products resulting from RNase digestion of the duplex RNAs shown in Figure 2.

digestion of the RNA duplex derived from DNA fragments containing a known mutation in the *p*53 gene confirming the presence of a mutation. In contrast, none of the assays performed to detect *ATM* mutations exhibited multiple bands, other than for samples obtained from eight patients in which the common G→A transition polymorphism at base 5557 in exon 39 (30–32) was detected. These results indicate that there were no *ATM* mutations present in the breast cancer patients tested.

Discussion

A series of 37 breast cancer patients was screened for germline mutations in the *ATM* gene. This work was accomplished using NIRCA, an assay that is complementary to the approaches which have been previously used to estimate *ATM* mutation frequency in breast cancer patients. No *ATM* mutations were detected in this study, which is consistent with the results obtained using other techniques [22–24]. Although the number of patients tested in this study was modest, our finding of no *ATM* mutations in a group of 37 patients provides a 95% upper confidence interval limit for *ATM* carriers among breast cancer patients of 7.8%.

NIRCA was used in this study for a variety of reasons:

(1) It has been shown that NIRCA is capable of detecting a variety of mutations, including point mutations as well as deletions and insertions, with a high level of sensitivity and accuracy [28, 29, 33–40];

(2) Relatively large target regions of at least 1000 bp can be screened in a single step. This is in contrast to other assays that are limited to much smaller target regions. This is critical for screening *ATM*, as

even the expressed portion of the gene is large, and the identified mutations are scattered throughout the gene;

(3) Using NIRCA, it is not necessary to possess any information as to potential sites for mutations. The entire coding sequence of the gene can be analyzed and therefore mutations at any point along the gene can be detected;

(4) The position of the bands on a gel provide a preliminary identification of the location for a mutation which facilitates DNA sequencing and analysis of sequencing data.

Acknowledgements

This research was supported by grant DAMD17-97-1-7208 from the Breast Cancer Research Fund of the Department of Defense and the Mount Sinai Breast Cancer Advisory Board. Barbara Ksienski is thanked for her technical assistance.

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